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MINIREVIEW

The War Against the Interferon-Induced dsRNA-Activated Protein Kinase: Can Viruses Win?

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INTRODUCTION

TO AVOID DECREASES in protein synthesis rates during infection, animal viruses down-regulate activity of the interferon (IFN) -induced, double-stranded (ds) RNA-activated protein kinase. Furthermore, there seems to be a good, but not perfect, correlation between the ability of a virus to down-regulate the kinase and its lack of susceptibility to IFN, suggesting this also may allow viruses to at least partially escape the antiviral effects of IFN. The purpose of this review is to describe the numerous and ingenious strategies employed by different virus groups to regulate kinase action. At the end I will discuss recent structure–function studies aimed at elucidating the molecular mechanisms underlying kinase activation and inhibition by viral-specific RNAs. For general reviews on IFN, IFN-induced genes, or the protein kinase in particular, the reader is directed to recent excellent reports.[1–6] In the present review, the dsRNA activated protein kinase will be referred to as P68 based on its M_r of 68,000 in human cells, although it also has been referred to by others as dsI, DAI, dsRNA-PK, eIF-2-PK_{ds}, and PI/eIF-2 kinase.[6–12] The only other species in which the kinase has been well characterized is mice, where the kinase has a M_r of 65,000[9,13]; however, recently we identified kinase homologues in bovine and monkey cells as well (G.N. Barber and M.G. Katze, unpublished).

The P68 protein kinase, recently molecularly cloned,[9] is a serine/threonine kinase characterized by two distinct kinase activities: the first involves an autophosphorylation (activation) reaction and the second a protein kinase activity on exogenous substrates.[10,14] Once activated by dsRNA, P68 phosphorylates its natural substrate, the α-subunit of protein synthesis eukaryotic initiation factor 2, eIF-2. Phosphorylation of the eIF-2 α-subunit blocks the eIF-2B-mediated exchange of GDP in the inactive eIF-2–GDP complex, with GTP required for catalytic utilization of eIF-2.[15–17] These events lead to limitations in functional eIF-2, which is an essential component of protein synthesis and is normally required to bind initiator Met-tRNA (*via* the ternary complex eIF-2–GTP–Met-tRNA) to the initiating ribosomal subunit before mRNA is bound.[18,19] Thus, activation of the kinase triggers a series of events that culminates in an inhibition of protein synthesis initiation. Since virus-specific RNAs synthesized during infection have the potential to activate P68,[20–26] viruses must down-regulate kinase activity to replicate and survive.

ADENOVIRUS

The regulation of the P68 protein kinase is best understood in the adenovirus system due to the seminal studies of the Shenk and Mathews laboratories.[27–33] Because these investigators recently wrote a comprehensive review of the P68 kinase and adenoviruses,[34] I will describe this system briefly and emphasize more recent work. The major advance in this field came with the description and characterization of the adenovirus mutant *dl*331.[33] During infection by this mutant, which is deficient in the synthesis of the adenovirus-encoded RNA polymerase III product, virus-associated RNA I (VAI RNA), all protein synthesis is blocked.[27,28,33] In the absence of VAI RNA, other virus-specific RNAs, presumably with double-stranded structures,[21] induced autophosphorylation of the kinase resulting in excessive eIF-2 α phosphorylation and a global inhibition of mRNA translation.[27,29] Several studies went on to show conclusively that VAI RNA, which itself has extensive double-stranded features,[35,36] functioned alone to block the dsRNA-induced activation of the P68 kinase[8,29,32] by forming a stable complex with the kinase.[8,12,37]

More recent and somewhat controversial studies have examined the structural features of VAI RNA that are critical for both function and binding to the P68 protein kinase. Mellits *et al.*,[12] in a series of *in vitro* experiments with VAI RNA mutants, found a lack of correlation between VAI RNA function and binding. These investigators concluded that there are two critical VAI RNA domains: the central domain, which is important for function, and the apical duplex, which is required for binding. A recent paper by Ghadge et al.[38] contradicted these data and demonstrated that mutations in the central domain (which is generally agreed to be vital for proper VAI RNA functioning) also eliminated binding. The discrepancies in these two reports can possibly be explained by the fact that Mellits et al.[12] based their conclusions solely on *in vitro* obser-

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vations whereas Ghadge et al.[38] examined binding of VAI RNA to P68 both *in vitro* and more importantly *in vivo* using reconstructed viruses containing the VAI RNA mutations. The findings that the central domain, which has relatively little double-stranded structure, is critical for P68 binding may have important implications for the mechanisms of VAI RNA action and how VAI RNA interacts with P68. Before closing this section it is relevant to note that the adenoviruses employ a second major strategy to avoid the antiviral effects of IFN, namely to inhibit, through action of the EIA gene products, IFN-inducible gene expression at the level of transcription.[39,40]

EPSTEIN-BARR VIRUS

Several years ago Bhat and Thimmappaya[41,42] observed that the Epstein-Barr virus-encoded EBER genes can, at least in part, functionally substitute for the adenoviral VA genes when present in adenovirus deletion mutants. EBER genes are similar to the VA genes in that they are of the same approximate size, are transcribed by RNA polymerase III, and their RNAs possess a similar degree of secondary structure with extensive double-stranded regions. The function of the EBER genes, has, however, remained elusive. Recently, Clemens and colleagues have postulated that EBER RNAs may function in a manner similar to VAI RNA. For example, it was shown that high concentrations of EBER-1 RNA can regulate protein synthesis in reticulocyte lysate possibly by blocking activation of the P68 protein kinase.[43] Subsequently, EBER-1 RNA was found to form a stable complex with P68 *in vitro* and this binding was dependent on the secondary structure, providing further evidence that these RNAs may work like VAI RNA.[44] It is important to note that, in contrast to the adenoviruses, no *in vivo* analysis has yet confirmed the role of the EBER RNAs in regulating the protein kinase. Now the story has been further complicated by the recent observations of Swaminathan *et al.*[45] who found that the lack of functional EBER genes in Epstein-Barr virus mutants did not adversely affect EBV replication or immortalization of cultured B cells.

INFLUENZA VIRUS

The first suggestion that influenza virus encoded a mechanism to regulate the P68 protein kinase came from our studies analyzing cells doubly infected with influenza virus and the adenovirus VAI RNA negative mutant *dl*331.[46,47] When *dl*331-infected cells were superinfected with influenza virus, a dramatic suppression of the protein kinase activity normally detected during *dl*331 infection was observed.[46,47] The P68 was immunopurified from doubly infected cells and the activity of the purified P68 measured by phosphorylation of exogenously added eIF-2. P68 kinase activity in the doubly infected cells was about fourfold lower than in cells infected by *dl*331 alone. These results strongly suggested that influenza virus encoded or activated a gene product that, analogous to the adenovirus VAI RNA, inhibited P68 autophosphorylation, thereby preventing the phosphorylation of eIF-2 and any resultant shutdown of protein synthesis.

We also were able to show the suppression of kinase activity in cells infected by influenza virus alone. This was directly demonstrated in our laboratory using the P68-specific monoclonal antibody, a high-affinity species-specific antibody that recognizes an epitope located at the very amino terminus of the human kinase.[26,48–50] Importantly, repression of the P68 kinase autophosphorylation correlated with a decrease in endogenous levels of eIF-2 α phosphorylation in cells infected by influenza virus alone. Suppression of kinase activity occurred as early as 2 h postinfection and required viral gene expression, specifically steps after primary transcription. When virus gene expression was restricted to primary transcription by carrying out infection in the presence of the protein synthesis inhibitor anisomycin, the P68 kinase activity was not suppressed but actually increased above control levels.[26] This suggested that influenza viral RNAs, by forming double-stranded structures, could activate the kinase. In keeping with the theme presented throughout this report, it made sense that influenza virus should encode a kinase repressor to counteract this activation.

To identify the putative influenza viral inhibitor, we developed an *in vitro* histone phosphorylation assay that quantitatively measured kinase inhibitory activity.[51] Using this assay and influenza virus-infected Madin-Darby bovine kidney cells as the starting material, the inhibitor was purified to near homogeneity. The final purified product, which had an MR of 58,000, inhibited both the autophosphorylation of P68 as well as phosphorylation of eIF-2 α when assayed in an *in vitro* reaction using only purified components. We tested for both protease and phosphatase activity but found neither associated with the purified inhibitor. More recently, we determined that the inhibition also was not due to the action of ribonuclease or ATPase nor did the inhibitor function by sequestering the RNA activators (Tae Gyu Lee and M. G. Katze, unpublished). Unexpectedly, Western blot analysis using virus-specific antibodies indicated that the purified repressor was a *cellular*- and not a viral-encoded protein. It has now been confirmed that indeed this repressor is cellular in origin and present at equivalent levels in uninfected and influenza virus-infected cells (T.G.L. and M.G.K., unpublished). To begin to decipher the molecular events controlling the regulation of the kinase by the 58 kD protein, we have recently obtained peptide microsequence information on the inhibitor. This has enabled us to prepare both peptide antibody and oligonucleotide probes to obtain a cDNA clone for the 58-kD protein using polymerase chain reaction (PCR) methodology. To our knowledge, this was the first report describing a cellular inhibitor of the P68 kinase. There are two other recent reports describing cellular regulators of P68 that appear to be distinct from that described in our laboratory. Saito and Kawakita[52] described an inhibitor partially purified from human FL cells with an MR greater than 160,000, whereas Judware and Petryshyn[53] have identified a 15-kD protein partially purified from 3T3-F442A cells and hypothesized to play a role in the ability of these cells to undergo adipose conversion. In related studies, Gupta and colleagues have described a cellular protein with a M_r of 67,000 that also may function to inhibit phosphorylation of eIF-2 α.[54]

Several questions may be posed, including why and how influenza virus activates the cellular 58-kD inhibitor during

infection. As stated, the kinase must be down-regulated to avoid negative effects on protein synthesis and also as a strategy to avoid the antiviral effects of IFN. Influenza virus is relatively insensitive to the antiviral effects of IFN unless the host cells possess the Mx gene[55] in which case viral transcription but not translation is blocked.[56] The more difficult question is how does influenza virus turn on the cellular 58-kD inhibitor? We did not observe kinase inhibitory activity in crude mock-infected extracts; however, after extracts were subjected to ammonium sulfate fractionation, P68 repressor activity was suddenly recoverable, essentially mimicking the situation following virus infection (T.G.L. and M.G.K., unpublished). One current hypothesis is that a factor or "anti-inhibitor" is dissociated from the 58-kD inhibitor during this high salt treatment (and also during virus infection). We have identified an anti-inhibitory activity in uninfected cells and are currently attempting to partially purify this factor from uninfected cells. The work on the cellular 58-kD inhibitor and its regulation should provide novel insights into the regulation of the kinase not only in virus-infected cells but also in uninfected cells. This may now be particularly important as there is accumulating evidence that the kinase, which is constitutively expressed in cells, plays a pivotal role in the regulation of cellular gene expression, in the absence of virus infection and IFN induction. For example, Petryshyn and colleagues have reported the kinase may be important in controlling growth arrest prior to differentiation into adipocytes.[57,58] Furthermore, it has been suggested that the P68 kinase may play a role in the heat-shock response[59] or the transcriptional regulation of select proto-oncogenes or IFN-β.[60,61]

PICORNAVIRUSES

A unique aspect of kinase regulation in picornavirus-infected cells is that these viruses appeared to lack mechanisms to block kinase activation. There was a dramatic increase in the autophosphorylation of P68 and phosphorylation of eIF-2α in cells infected by poliovirus,[20] mengovirus,[62] and encephalomyocarditis virus (EMC).[63] Although mengovirus completely lacked a plan to regulate the activated kinase,[62] EMC virus caused a change in the subcellular localization of the kinase and perhaps in this way minimized the consequences of an activated P68.[63]

Poliovirus, on the other hand, employed an uncommon strategy to deal with a protein kinase that had been activated by virus-specific dsRNAs: the kinase was dramatically degraded during infection.[20] Despite P68 degradation, we and others observed that the levels of eIF-2 α phosphorylation still increased in poliovirus-infected cells in response to an activated P68.[20,64] It remains to be determined why poliovirus encoded a mechanism to degrade P68 and not to block the activation step like many other viruses. One possibility is that enhanced α phosphorylation early after infection may help play a role in the host protein synthesis shut-off.[5] Nevertheless, it is certain that without significant P68 proteolysis, eIF-2 α phosphorylation would increase to the degree that even viral protein synthesis could not occur, a scenario unacceptable to the virus.

It is intriguing that poliovirus induced the degradation of two proteins which have important roles in translational regulation: P68, the dsRNA-activated protein kinase, and P220, an essential component of the cap binding protein complex.[65] It is mainly due to P220 degradation that the uncapped poliovirus mRNAs have an advantage over cellular RNAs and are efficiently translated in virus-infected cells.[5,65,66] Although the poliovirus-encoded 2A protease indirectly induced the cleavage of P220,[67] recent work from our laboratory suggested that neither 2A nor the poliovirus protease 3C work directly to degrade P68 (T. Black and M.G. Katze, unpublished). An *in vitro* assay has since been developed to purify and characterize the responsible protease biochemically. Preliminary data suggest that the protease is insoluble and may be composed of both an RNA and protein component, because activity is inhibited by the action of both trypsin and ribonuclease. These experiments are ongoing and hopefully will elucidate the regulatory mechanisms underlying P68 degradation in poliovirus-infected cells.

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

The earliest reports suggesting the involvement of the P68 kinase with HIV gene expression showed that fusion of the Tat-responsive region in the 5′ untranslated region of HIV mRNAs (termed TAR) to a heterologous mRNA (CAT) exhibited *cis*- and *trans*-inhibitory effects on mRNA translation *in vitro*.[22,68] These inhibitory effects, which were dependent on the secondary structure of the TAR region, could be reversed by addition of the recycling factor, eIF-2B, and were prevented by high concentrations of poly(I):poly(C). Moreover, synthetic RNAs containing TARCAT sequences stimulated the autophosphorylation of the P68 kinase and phosphorylation of eIF-2 α in cell-free extracts.[22] In confirmation of these data, Sengupta and Silverman[24] found that affinity resins, consisting of HIV-1 leader RNA covalently bound to cellulose, activated the P68 kinase. Taken together, these studies strongly suggested that the TAR region could activate P68 *in vitro*.

To address the question whether TAR RNAs could activate the kinase *in vivo*, we monitored the ability of TAR containing poly(A)$^+$ RNAs from HIV-1-infected cells to activate P68. Not only did these RNAs induce kinase activation but we also found that TAR-containing RNAs formed a stable complex with the P68 protein kinase.[25] Both efficient binding to and activation of the protein kinase were dependent on the integrity of the RNA stem structure. Although it has been suggested that TAR-induced activation may be caused by dsRNA contaminants in the RNA preparation,[69] this is unlikely for the following reasons: (i) nucleotide substitutions predicted to disrupt base-pairing in the TAR secondary structure were poor activators of the kinase compared with RNAs with the wild-type TAR sequences or with compensatory mutations; (ii) when the chromatographic analysis of TAR RNAs on CF11 cellulose was performed, the activating TAR-containing RNAs did not elute in the fraction expected to contain contaminating dsRNAs; and (iii) most importantly, RNAse III, a ribonuclease highly selective for potentially contaminating perfect dsRNA structures, did not affect the TAR-mediated activation of the kinase. We did find that, although low concentrations of TAR RNAs activated P68, high concentrations inhibited kinase activation[25] as reported by others.[69]

HIV-1 has evolved mechanisms to prevent the TAR-mediated activation of the kinase from disrupting viral gene expression. In collaboration with Nahum Sonenberg's laboratory, we found that productive infection by HIV-1 resulted in a significant decrease in the amounts of the P68 protein kinase. Furthermore, the steady-state levels of P68 were reduced in IFN-treated HeLa cell lines stably expressing Tat, as compared to the amount of the kinase in IFN-treated control HeLa cells.[70] Thus, the potential translational inhibitory effects of the TAR RNA region mediated by activation of the kinase may be down-regulated by Tat during productive HIV-1 infection. It is unlikely that down-regulation of the kinase by Tat confers a resistance of HIV-1 to the general antiviral effects of IFN. Indeed, IFN has been shown to block viral replication at both early and late stages, depending on the cell type, although the mechanisms are poorly understood (for recent reviews see refs. 71 and 72). Since HIV-1 viral protein synthesis has not yet been shown to be inhibited by IFN treatment, it is tempting to speculate that Tat may prevent IFN and the kinase from adversely affecting mRNA translation in HIV-1–infected cells.

Finally, it is relevant to note that activity of another dsRNA-dependent IFN-inducible gene, the 2′-5′A synthetase, has been implicated in the regulation of HIV-1 gene expression. Productive HIV-1 infection correlated with activation of the 2′-5′A synthetase,[73] which, in turn, activates RNase L (a latent endoribonuclease; see refs. 3 and 7). Although not proven, it is possible that activation of this system may result in the degradation of viral and cellular RNAs that has been observed in HIV-1–infected cells.[73–76] Interestingly, TAR RNAs were found to bind and activate the synthetase[24] and the Tat protein was found to block this activation.[77] Thus, the TAR sequence in the 5′ untranslated region of HIV-1 mRNAs may play a negative role in viral gene expression *via* activation of the P68 kinase and 2′-5′A synthetase. It is an interesting possibility that up-regulation in amounts of these proteins by administration of IFN may thus prolong viral latency. However, the switch from latency to active viral replication would necessitate a mechanism to depress activity of both enzymes and this function may be provided by the HIV-1 Tat gene product.

REOVIRUSES

Imani and Jacobs[78] reported that the P68 protein kinase was inhibited in reovirus-infected cells due to the action of the reovirus serotype 1 sigma 3 protein. Because the action of sigma 3 protein, identified as a dsRNA-binding protein, can be reversed by adding high concentrations of dsRNA to *in vitro* reactions, it likely sequesters RNA activators of the kinase. It also has been shown that the sigma 3 protein can enhance translation of a reporter gene when both are transfected into COS cells, possibly by preventing P68 activation.[79] Before making definitive conclusions about reovirus-induced regulation of P68, however, it is essential to examine the phosphorylation states of the kinase and eIF-2 α *in vivo*, in cells infected with both wild-type reovirus and mutants defective in the sigma 3 protein if possible. It would not be surprising if regulation of the kinase was required during infection because the reovirus dsRNAs are potent activators of the kinase and even single-stranded reovirus mRNAs have varying abilities to activate P68, at least *in vitro*.[23]

VACCINIA VIRUS

Like reovirus, vaccinia virus down-regulated the P68 kinase by encoding a protein product that sequestered dsRNA activators. Pioneering work had shown that the ability of vaccinia to replicate in IFN-treated cells correlated with the presence of a virally coded factor which blocked the autophosphorylation of P68 and phosphorylation of eIF-2 α *in vitro*.[80–82] This factor was referred to as SKIF, for specific kinase inhibitory factor. SKIF was expressed relatively early after infection and its action can be neutralized by addition of excess dsRNA.[82] Akkaraju *et al.*[11] examined the effects of a partially purified SKIF in rabbit reticulocyte extracts and found that the factor inhibited eIF-2 α phosphorylation by the P68 kinase and prevented the dsRNA-induced inhibition of protein synthesis in these lysates. Watson *et al.*[83] have now identified a vaccinia virus-encoded 25,000-dalton dsRNA binding protein, which copurified with kinase inhibitory activity and which was made with kinetics similar to this activity. It still remains to be determined whether this protein is identical to SKIF and whether kinase regulation actually occurred *in vivo* because all the conclusions reached thus far have been based on *in vitro* studies.

Exciting new results from the laboratory of Paoletti and colleagues[84] suggested that vaccinia virus may have evolved an additional strategy to avoid the antiviral effects of IFN. They have identified an 88-amino-acid-long open reading frame, designated as K3L, which has 28% identity to the α-subunit of eIF-2. Deletion analysis suggested a correlation between resistance to IFN and the presence of the K3L gene product. Although the exact mechanisms by which this occurred must be worked out, it has been hypothesized that K3L, which lacks the key phosphorylation site found in the native subunit, conferred IFN resistance by complexing with the P68 protein kinase and blocking phosphorylation of the *bona fide* cellular eIF-2 α.[84]

STRUCTURE–FUNCTION ANALYSIS OF THE P68 PROTEIN KINASE

Despite the many studies described above, we still know little about the molecular mechanisms of activation and repression of the P68 protein kinase by virus-specific RNAs. P68 does not possess the ribonucleoprotein consensus sequences[85] typical of RNA-binding proteins, although the sequence indicates that the kinase is a hydrophilic protein that may account for its ability to bind dsRNA.[9] In this section I would like to summarize briefly some work on mutational analysis from our laboratory and others that examined the binding of dsRNA activators (native reovirus dsRNAs and synthetic dsRNAs) and the adenoviral VAI RNA inhibitor to P68. One objective has been to determine whether VAI RNA functioned by competing with activator dsRNAs for identical sites on the kinase.

The data thus far have revealed that the amino, noncatalytic half of the kinase is important for the binding of both activator

and repressor RNAs.[49,86–88] Truncated molecules that lacked the kinase carboxyl region containing all the classic catalytic domains[89] efficiently bound VAI RNA and reovirus dsRNA[49] as well as poly(I):poly(C).[87,88] As might be predicted, P68 molecules containing only the carboxyl terminus failed to bind these RNAs[87] (G.N. Barber and M.G. Katze, unpublished). Exactly what regions of the amino terminus are required for binding remains unclear, although the emerging picture suggests that much of this region is critical and that any tampering with this part of the molecule will disrupt dsRNA and VAI RNA binding. For example, we now have data from deletion analysis that, minimally, amino acids 1–97 and 155–242 are essential for reovirus dsRNA and VAI RNA binding, but we cannot rule out that other regions are involved. Our results are in general agreement with the recent paper of Patel and Sen[88] who found that two regions of the kinase, encompassing amino acids 1–35 and 129–170, are critical for binding the synthetic dsRNA poly(I):poly(C). For the present, all these data must be interpreted with some caution until finer mutagenic analysis is performed because deletions and truncations may well alter the structure of the kinase molecules. Finally, regarding the question about whether activator dsRNAs and VAI RNA are binding to identical site: We have thus far found a good correlation, but again a more precise mapping with many additional mutants is required before a definitive answer is obtained.

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